Islet1 Derivatives in the Heart Are of Both Neural Crest and Second Heart Field Origin

Kurt A. Engleka,* Lauren J. Manderfield,* Rachael D. Brust, Li Li, Ashley Cohen, Susan M. Dymecki, Jonathan A. Epstein

Rationale: Islet1 (Isl1) has been proposed as a marker of cardiac progenitor cells derived from the second heart field and is utilized to identify and purify cardiac progenitors from murine and human specimens for ex vivo expansion. The use of Isl1 as a specific second heart field marker is dependent on its exclusion from other cardiac lineages such as neural crest.

Objective: Determine whether Isl1 is expressed by cardiac neural crest.

Methods and Results: We used an intersectional fate-mapping system using the RC::FrePe allele, which reports dual Flpe and Cre recombination. Combining Isl1Cre/+, a SHF driver, and Wnt1::Flpe, a neural crest driver, with Rc::FrePe reveals that some Isl1 derivatives in the cardiac outflow tract derive from Wnt1-expressing neural crest progenitors. In contrast, no overlap was observed between Wnt1-derived neural crest and an alternative second heart field driver, Mef2c-AHF-Cre.

Conclusions: Isl1 is not restricted to second heart field progenitors in the developing heart but also labels cardiac neural crest. The intersection of Isl1 and Wnt1 lineages within the heart provides a caveat to using Isl1 as an exclusive second heart field cardiac progenitor marker and suggests that some Isl1-expressing progenitor cells derived from embryos, embryonic stem cell cultures, or induced pluripotent stem cells may be of neural crest lineage. (Circ Res. 2012;110:00-00.)

Key Words: myocardial lineages • second heart field • neural crest • heart development

The recent discovery of the second heart field (SHF) has redefined the understanding of mammalian cardiac development.1 While cells of the first heart field (FHF) form the early cardiac tube, the SHF contributes additional cells to the maturing heart through midgestation, ultimately forming large portions of the right ventricle, outflow tract, and atria.2–4 SHF precursors have been characterized by expression of the homeobox gene Islet1 (Isl1).2 Isl1 has been used to isolate putative cardiac progenitors from embryonic stem (ES) cell and induced pluripotent stem (iPS) cell cultures for the purpose of expansion and possible therapeutic application.5–10 In vitro studies and in vivo fate-mapping experiments suggest that Isl1 precursors are tripotential and give rise to endothelium, smooth muscle, and cardiac muscle.7,9 Although SHF-specific enhancer elements from several genes have been identified by the analysis of transgenic mice,3,11 no other specific markers for SHF precursors have been reported, emphasizing the importance that Isl1 has played as a specific marker of the SHF in cardiac and stem cell biology.

Surprisingly, inactivation of various factors in SHF using Isl1Cre/+ has resulted in mice with congenital heart defects involving the outflow tract (OFT) and aortic arch arteries that are strikingly similar to abnormalities produced by gene manipulation using Wnt1::Cre or Pax3Cre+/+, which drive expression in cardiac neural crest.12–14 This has led to the suggestion that SHF signals to neural crest via cell–cell interactions or secreted factors. However, an alternative hypothesis is that Isl1Cre/+ is not restricted to SHF within the developing heart. To address whether Isl1Cre/+ may also be expressed in the Wnt1 lineage of cardiac neural crest in addition to lineages of the SHF, we utilized a dual-fluorescent reporter allele that extends previously established recombinase-based intersectional strate-

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The dual fate-mapping approach uses a recently developed reporter mouse, RC::FrePe (R.D. Brust and S.M. Dymecki, unpublished data, and see Bang et al18) that activates expression of enhanced green fluorescent protein (eGFP) within cells that have expressed both Flpe and Cre at any time and in any order within their lineage. In cells that have expressed Flpe alone, mCherry is expressed, while Cre activity alone is not reported. Thus, RC::FrePe indicates an intersectional population between expression domains as defined by expression of separate Flpe and Cre drivers.

Methods

A schematic of the RC::FrePe allele can be found within a summary of intersectional fate-mapping strategies and methods.19

Histology and Immunohistochemistry

Samples were harvested, fixed overnight in 2% formaldehyde, and subsequently dehydrated through an ethanol series. Samples were then paraffin embedded and sectioned. For whole-mount staining of LacZ expression, samples were lightly fixed with 2% formaldehyde for 30 minutes and then incubated overnight with X-Gal. Antibodies used for immunohistochemistry were anti-GFP goat polyclonal (Abcam, Cambridge, MA), anti-mCherry goat polyclonal (Clontech, Mountain View, CA), anti-GFP goat polyclonal (Abcam, Cambridge, MA), and anti-αSMA mouse monoclonal 1A4 (Sigma-Aldrich, St. Louis, MO). Quantitation of fluorescence was performed using ImageJ software (National Institutes of Health, Bethesda, MD).

Genotyping information can be found in the Online Data Supplement.

Results

To validate the reporter activity of RC::FrePe in neural crest derivatives, we used 2 neural crest drivers, Wnt1::Flpe20,21 and Pax3Cre+22 to assess recombination and reporter activity (Online Figure IA through IO). Direct fluorescence of postnatal (P) day 0 Wnt1::Flpe Pax3Cre+/+:RC::FrePe hearts demonstrates eGFP expression within the outflow tract where neural crest derivatives reside (Online Figure IM).22 Subsequent immunofluorescence confirms eGFP protein expression within the tunica media of the mature OFT, as expected for neural crest derivatives (Online Figure IO). Relatively few mCherry-labeled cells remain after dual Flpe/Cre recombination, suggesting that most cardiac neural crest cells that expressed Wnt1::Flpe also expressed Pax3Cre+/+ (Online Figure IN). An average of fluorescence from 9 stained Wnt1::Flpe Pax3Cre+/+:RC::FrePe outflow tract sections revealed that 8.4%±3.6% (mean±SD of total staining was mCherry positive, while the majority, 91.6%±3.5%, was eGFP positive. Thus, the indicator allele RC::FrePe is sensitive to dual recombination, consistent with previous findings (R.D. Brust, and S.M. Dymecki, unpublished data, and see Bang et al18). We did not detect any leakiness of eGFP or mCherry expression in these studies (Online Figure IB through IE).

We crossed Isl1Cre+/+23,24 mice with Wnt1::Flpe and RC::FrePe mice to detect overlap in Isl1- and Wnt1-expressing populations (Figure 1A through IO). In the absence of Flpe and Cre, only background fluorescence was observed in embryonic (E) day 12.5 embryos (Figure 1A through IE). When Wnt1::Flpe was expressed in the presence of RC::FrePe, mCherry expression was observed by direct fluorescence in the craniofacial region populated by neural crest (Figure 1G, arrowhead) and in dorsal root ganglia (DRG, Figure 1G, arrows). The eGFP was not detected (Figure 1H). The mCherry+ cells were detected by immunofluorescence in sections through the OFT endocardial cushions that are populated by cardiac neural crest by E12.5 (Figure 1I), while eGFP was not detected (Figure 1J). In the presence of both Wnt1::Flpe and Isl1Cre+, mCherry expression persisted in craniofacial mesenchyme populated by cranial neural crest (Figure 1L, arrowhead), and eGFP fluorescence was observed in DRGs (Figure 1M, arrows), consistent with the known expression of Isl1 in neural crest derived DRGs.25 Immunofluorescence confirmed expression of both mCherry (Figure 1N) and eGFP (Figure 1O) in sections through the endocardial cushions of the OFT, indicating that at least some cardiac neural crest derivatives in the heart have expressed Isl1Cre+ at some time in their development.

A transgenic mouse with an enhancer element derived from the Mef2c gene directing expression of Cre recombinase, Mef2c-AHF-Cre, is widely used to label SHF derivatives.11 We crossed this mouse with Wnt1::Flpe and RC::FrePe but did not detect any evidence for overlap of expression domains at E12.5 (Figure 1P through 1T). This result indicates that Mef2c-AHF-Cre may be more restricted to SHF precursors than is Isl1Cre+/+ and also serves as an important negative control to minimize the chance that leaky expression explains the presence of eGFP in Wnt1::Flpe; Isl1Cre+/+::RC::FrePe embryos.

Analysis of P0 hearts of Wnt1::Flpe; Isl1Cre+/+::RC::FrePe, and Wnt1::Flpe;Mef2c-AHF-Cre; RC::FrePe pups confirms the stark difference between Isl1Cre+/+ and Mef2c-AHF-Cre (Figure 2A through 2J and Online Figure II). Both mCherry and eGFP are evident in the OFT of P0 Wnt1::Flpe; Isl1Cre+/+::RC::FrePe hearts (Figure 2B and 2C) visualized by, and eGFP immunofluorescence is evident in the tunica media of the great vessels (Figure 2D) and in the
leaflets of the aortic valve (Figure 2E). The eGFP expression is not detected in Wnt1::Flpe; Mef2c-AHF-Cre; RC::FrePe hearts (Figure 2H through 2J). The areas of fluorescence marked by either Isl1 Cre/H11001 or Mef2c-AHF-Cre with Rc::FrePe are within the fate-mapped domains marked by these drivers using R26LacZ/H11001 (Online Figure II). The eGFP expressing derivatives of Isl1Cre/+ and Wnt1-expressing precursors are able to differentiate into smooth muscle, as evidenced by coexpression of eGFP and smooth muscle actin (SMA) in the proximal aorta of Wnt1::Flpe; Isl1Cre+/+;RC::FrePe pups (Figure 2K through 2M, arrowheads). The eGFP+ cells are observed near the branchial arches of Wnt1::Flpe; Isl1Cre+/+;RC::FrePe embryos as early as day E10.5 and within the aortic sac (Online Figure III).

**Discussion**

These results indicate that Isl1Cre/+ is not restricted to SHF precursors of the mature heart. Rather, Isl1Cre/+ labels both SHF precursors and also at least some cardiac neural crest cells. Isl1 is known to be expressed by other neural crest derivatives, including DRG and cardiac ganglia, but the demonstration of Isl1Cre/+ expression by cardiac neural crest precursors demands reevaluation of its use as an SHF driver and reinterpretation of some prior studies.

For example, both neural crest and SHF can give rise to smooth muscle. Given our results, it is now unclear whether some or all Isl1 derivatives in the OFT that express smooth muscle markers are neural crest or SHF derived.2,23,24,26 Our data suggest that at least some are of neural crest origin. Isolation of Isl1-expressing cells from ES or iPS cultures, or from embryos, for the purpose of expanding SHF precursors (see references listed in supporting information in the Online Data Supplement)2,6,7,9,23 may actually result in the expansion of neural crest cells. Studies in which Isl1Cre/+ has been used to manipulate gene expression in the SHF, and which have resulted in aortic arch and OFT defects, may need to be reexamined for the possibility of cell autonomous neural crest effects. Experimental approaches using multiple or alternative SHF drivers, such as Mef2c-AHF-Cre, and use of dual and intersectional fate-mapping approaches, such as the one described here, may define cardiac cell origins and fates more clearly than would using the Isl1 marker alone. Our
studies emphasize the need for the identification of more specific molecular markers of SHF precursors.

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Disclosures
None.

References


**Novelty and Significance**

**What Is Known?**

- Within the developing heart field, *Isl1* is postulated as a selective marker of cardiac progenitor cells derived from the second heart field.
- The specificity of *Isl1* as a marker for second heart field is critical to lineage tracing, gene inactivation, and differentiation analyses.
- *Isl1* derivatives include cells populating the outflow tract, an area patterned and formed by derivatives of the cardiac neural crest and second heart field.

**What New Information Does This Article Contribute?**

- A reporter mouse *RC::FrePe* allows identification of cells undergoing dual Flpe- and Cre-mediated recombination and sensitively indicates intersection between lineages marked by separate Flpe and Cre drivers.
- *Isl1* is not restricted to second heart derivatives in the heart but is also expressed by a subset of cardiac neural crest cells.
- The intersectional population revealed by *Wnt1::Flpe; Isl1Cre*/*Rc::FrePe* resides in the cardiac outflow tract and includes smooth muscle cells of the tunica media of the aorta and pulmonary artery.
- Dual fate mapping using an alternative second heart field driver, a *Mef2c* enhancer regulating Cre, does not overlap with neural crest.

*Isl1* is postulated as a marker of cardiac progenitor cells from the second heart field. Numerous studies suggest that *Isl1*+/cells represent tripotential precursors of differentiated cardiac tissues, including smooth muscle, cardiac muscle, and endothelial cells. Using Flpe- and Cre-mediated dual fate mapping, we show that *Isl1* is not restricted to second heart derivatives in the heart but that it is also expressed by a subset of cardiac neural crest cells. Thus, some *Isl1* cardiac derivatives may be neural crest–derived rather than a multipotent second heart field precursor. These findings suggest that results based on *Isl1* fate mapping should be interpreted with caution and emphasize the need for additional cardiac lineage tools and markers.
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Data Supplement (unedited) at:
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Supplemental Material

Detailed Methods

Mice

All animal protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Mice were maintained on a mixed genetic background. \( Isl1^{Cre/+}_{1,2} \), \( Pax3^{Cre}_{3} \), \( Mef2c-AHF-Cre \) \(^4\) and \( R26^{LacZ/+}_{5} \) mice were genotyped as previously described. \( Wnt1::Flpe \) mice \(^6,7\) were genotyped using the following primers as described: \(^7\)

Forward: 5' GGTCCTGGTTGTCAGTTTGTG 3'
Reverse: 5' TCCCTTATCTGCTTCTTCCGATG 3'

The dual reporter allele \( RC::FrePe \) was genotyped with the following primers for the Rosa26 locus:

Forward: 5' CACTTGCTCTCCCAAAGTCG 3'
Wild-type Reverse: 5' TAGTCTAACTCGCGACACTG 3'
Mutant Reverse: 5' GTTATGTAACGCGGAACTCC 3'
Supplemental Figures and Figure Legends

Online Figure I. Validation of the dual reporter \textit{RC::FrePe} mouse in neural crest. \textit{RC::FrePe} is knocked into the \textit{Rosa26} locus as per previously designed intersectional reporter alleles.\textsuperscript{6, 8, 9} A-E, Postnatal (P) day 0 control \textit{RC::FrePe} whole hearts (A-C) and cross sections through the outflow tract (D, E). mCherry and eGFP are not detected. F-J, P0 hearts from \textit{Wnt1::Flpe; Pax3\textsuperscript{+/+}; RC::FrePe} embryos. mCherry expression is detected in the outflow tract (G, \textit{arrowhead}) and is confirmed by immunofluorescence of cross sections (I). eGFP is not detected (J). K-O, P0 hearts from \textit{Wnt1::Flpe; Pax3\textsuperscript{Cre/+}; RC::FrePe} pups. Strong eGFP expression is now evident in the outflow tract (M, \textit{arrowhead, and O}) while only trace amounts of mCherry expression are detected (L, N). Scale bars: 100\textmu m. BF-Bright field. IF-Immunofluorescence
Online Figure II. *Mef2c-AHF-Cre* and *Isl1\textsuperscript{Cre/+}* derivatives in newborn hearts include the area of *RC::FrePe* dual-reporter expression. (A-C) Post-natal day 0 (P0) *Wnt1::Flpe; Mef2c-AHF-Cre; RC::FrePe* heart showing mCherry fluorescence in the outflow tract (B, arrow). eGFP is not detected (C, arrow). D, P0 *Wnt1::Flpe; Mef2c-AHF-Cre; R26\textsuperscript{LacZ/+}* whole-mount X-Gal-stained hearts demonstrating *LacZ* expression in the outflow tract in comparable regions of the outflow tract highlighted in A-C (D, arrow). (E-G) P0 *Wnt1::Flpe; Isl1\textsuperscript{Cre/+}; RC::FrePe* hearts showing mCherry (F, arrow) and eGFP (G, arrow) fluorescence in the cardiac outflow tract. H, P0 *Wnt1::Flpe; Isl1\textsuperscript{Cre/+}; R26\textsuperscript{LacZ/+}* whole-mount X-gal stained heart demonstrating *LacZ* expression in the outflow tract in comparable regions of the outflow tract highlighted in E-G (H, arrow).
Online Figure III. Dual fate mapping identifies \( Isl1^{Cre/+} / Wnt1::Flpe \)-derived cells in the heart at E10.5. 

A-E, E10.5 \( Wnt1::Flpe; Isl1^{+/+}; RC::FrePe \) embryos (A-C) and immunofluorescence (IF) for mCherry (D) and eGFP (E) of cross sections through the region of the developing cardiac outflow tract (D,E). 

\( Wnt1::Flpe \)-derived craniofacial neural crest (B, arrowhead) and dorsal root ganglia (B, arrows) express mCherry, which is also detected by IF in the developing outflow tract (D). 

F-J, \( Wnt1::Flpe; Isl1^{Cre/+}; RC::FrePe \) embryos. mCherry is detected in craniofacial mesenchyme (G, arrowhead) and eGFP is now expressed by dorsal root ganglia (H, arrows). mCherry (I) and eGFP (J) are both detected by IF in the developing outflow tract. 

K-M, Higher magnification of the \( Wnt1::Flpe; Isl1^{Cre/+}; RC::FrePe \) embryo shown in F-H. mCherry is detected in the first (I) and second (II) pharyngeal arches (L) while eGFP is expressed by cells near the pharyngeal arches (M, arrows). Scale bars: 100\( \mu \)m.
Supplemental Tables and supporting information

Reports Utilizing Isl1 for Identification of Second Heart Field Precursors


Supplemental References

Islet1 Derivatives in the Heart Are of Both Neural Crest and Second Heart Field Origin: Correction

In the article that appears on page 922 of the March 30, 2012 issue, the printed version of Figure 1 is a poor representation of the original data. The online version is a more accurate reproduction.

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